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Estrogen Effects after a Crush Muscle Injury and Acute Exposure to Hypobaric Hypoxia

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14. ABSTRACT

Aeromedical evacuation, also known as en route care, is a critical and successful strategy for treating soldiers wounded on the battlefield. However, because the aeromedical platform maintains a cabin pressure equivalent to an altitude of 8,000 feet, which is considered high altitude, and lacks abundant supplemental oxygen systems, en route care exposes wounded soldiers to low oxygen content. To ensure wounded soldiers experience a complete recovery, a greater understanding of the effects of hypobaric hypoxia (HH) on injured or damaged tissue is important. The overall objectives of this 4-year project were to (a) create experimental models for studying the effect of HH on muscle recovery and (b) test the effect of estrogen, as a countermeasure, on HH effects on muscle recovery. The major results were as follows: (a) a single exposure to HH for 8–9 hours did not activate the genes and cells of inflammation in healthy skeletal muscle; (b) after an acute closed crush muscle injury, which induced significant inflammation alone, exposure to HH for 8–9 hours led to a reduction in a specific subpopulation of white blood cells, macrophages, at 32 hours post-injury; and (c) estrogen did not counteract the suppressive effect of HH on white blood cells, but did upregulate muscle-specific factors. In conclusion, the occurrence of en route care at 24 hours after an acute muscle injury may hinder the inflammatory response. Although estrogen may not be an effective countermeasure for this response, estrogen may promote muscle regeneration.

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1.0 EXECUTIVE SUMMARY

Aeromedical evacuation, also known as en route care, is a critical and successful strategy for treating soldiers wounded on the battlefield. However, because the aeromedical platform maintains a cabin pressure equivalent to an altitude of 8,000 feet, which is considered high altitude, and lacks abundant supplemental oxygen systems, en route care exposes wounded soldiers to low oxygen content. To ensure wounded soldiers experience a complete recovery, a greater understanding of the effects of hypobaric hypoxia (HH) on injured or damaged tissue is important.

The overall objectives of this project were to (a) create experimental models for studying the effect of HH on muscle recovery and (b) test the effect of estrogen, as a countermeasure, on HH effects on muscle recovery. This 4-year project consisted of two phases, and each phase consisted of technical milestones. In Phase 1, there were four technical milestones, M5 Objective 1/M8, M5 Objective 2/M9, M13A, and M11. The Phase 1 research questions are listed below:

Research Question 1 (M5 Objective 1/M8)

Will acute HH exposure independently enhance neutrophil and macrophage infiltration and pro-inflammatory cytokine production and alter gene expression in the lower extremity (uninjured) muscle of mice?

Research Question 2 (M5 Objective 2/M9)

Will crush injury induce neutrophil and macrophage infiltration and pro-inflammatory cytokine production and alter gene expression in the lower extremity muscle of mice in a normobaric environment?

Research Question 3 (M13A)

Will 4 weeks of estrogen treatment induce an altered metabolic response in comparison with 2.5 weeks of estradiol treatment?

Research Question 4 (M11)

Will acute HH exposure enhance neutrophil and macrophage infiltration and pro-inflammatory cytokine production and alter gene expression in lower extremity crush-injured muscle of mice?

In Phase 2, there were three technical milestones that were later combined into one milestone, known as M13/M15/M17. Research question 5 is as follows:

Will estrogen promote neutrophil and macrophage infiltration, cytokine production, and inflammation-related gene expression in lower extremity crush-injured muscle of mice exposed to acute HH?

In this final report, three published articles provide the methods and results of research questions 1 and 2. Individual sections of this report address research questions 3, 4, and 5.

The overall results are as follows:

(a) A single exposure to HH for 8–9 hours did not activate the genes and cells of inflammation in healthy skeletal muscle.
(b) After an acute closed crush muscle injury, which induced significant inflammation alone, exposure to HH for 8–9 hours led to a reduction in a specific subpopulation of white blood cells, macrophages, at 32 hours post-injury.
(c) Estrogen did not counteract the suppressive effect of HH on white blood cells in crush-injured muscle.
(d) The white blood cell (both neutrophils and macrophages) response to an acute closed crush muscle injury and HH appeared to be dampened in ovariectomized subjects with estrogen alone or without estrogen and progesterone.
(e) The completion of muscle regeneration after crush injury and HH appeared to be delayed in ovariectomized subjects without estrogen and progesterone.

In conclusion, the occurrence of en route care at 24 hours after an acute muscle injury may hinder the inflammatory response; however, estrogen may not be an effective countermeasure for this response. In addition, ovariectomy may have secondary effects that lead to an alteration in the inflammatory response and muscle regeneration.

From a military perspective, future studies should address whether (a) the occurrence of en route care at 24 hours after an acute muscle injury affects muscle regeneration; (b) a more complex experimental model (e.g., hemodynamic changes + acute muscle injury + HH for 8-16 hours), which is more representative of the field condition, induces a greater suppressive effect on white blood cells; (c) a high estrogen dose is an effective countermeasure for the suppressive effect of HH; and (d) other substances that promote macrophage infiltration into injured tissue are effective HH countermeasures.

2.0 INTRODUCTION

Aeromedical evacuation or en route care has been available since World War II [1] and has “revolutionized” [2] the care of wounded troops. However, terrorist attacks in 1998 and 2000 and recent conflicts in the Middle East have increased the use of and demand for aeromedical evacuation of wounded U.S. troops [1]. In 2000, more than 25% of the sailors who were critically injured from a terrorist attack on the U.S.S. Cole were air evacuated from Yemen to Djibouti and then to Germany [3]. Between October 2001 and March 2011, 86,000 soldiers were air evacuated for an injury or disease [4]. In addition, en route care is also a successful strategy. For example, survival rate after combat trauma is now 91–99% [5]. Therefore, aeromedical evacuation is critical to the health and recovery of U.S. troops.

A potential challenge of en route care is hypobaria. Typically, the cabin of fixed-wing aircraft used for en route care is pressurized to an altitude of ~8,000 feet [2]. At this altitude, the blood oxygen saturation of a healthy person declines to 90%, which is 8-10% below normal [2]. At an altitude of 8,000 feet, the oxygen saturation of critically wounded soldiers will drop even lower [3]. Therefore, the environment of en route care provides a reduced oxygen supply for injured and uninjured tissues.

A reduced oxygen supply from high altitude is known as hypobaric hypoxia (HH). Our understanding of the effect of HH on injured or damaged tissue is important in case we need to intervene to ensure that wounded soldiers experience a complete recovery. For this reason, the objectives of this project were to (a) create experimental models for studying the effect of HH on

muscle recovery and (b) test the effect of estrogen, as a countermeasure, on HH effects on muscle recovery. This 4-year project consisted of two phases, and each phase consisted of technical milestones. In Phase 1, there were four technical milestones: M5 Objective 1/M8, M5 Objective 2/M9, M13A, and M11. The Phase 1 research questions are listed below:

Research Question 1 (M5 Objective 1/M8)

Will acute HH exposure independently enhance neutrophil and macrophage infiltration and pro-inflammatory cytokine production and alter gene expression in the lower extremity (uninjured) muscle of mice?

Research Question 2 (M5 Objective 2/M9)

Will crush injury induce neutrophil and macrophage infiltration and pro-inflammatory cytokine production and alter gene expression in the lower extremity muscle of mice in a normobaric (NB) environment?

Research Question 3 (M13A)

Will 4 weeks of estradiol treatment induce an altered metabolic response in comparison with 2.5 weeks of estradiol treatment?

Research Question 4 (M11)

Will acute HH exposure enhance neutrophil and macrophage infiltration and pro-inflammatory cytokine production and alter gene expression in lower extremity crush-injured muscle of mice?

In Phase 2, there were three technical milestones that were later combined into one milestone, known as M13/M15/M17. Research question 5 is listed below:

Will estrogen attenuate neutrophil and macrophage infiltration and pro-inflammatory cytokine production and alter gene expression in lower extremity crush-injured muscle of mice exposed to acute HH?

In this final report, three published articles provide the methods and results of research questions 1 and 2:

- St Pierre Schneider B, Moonie S, Fulkerson ND, Nicholas J, Bammler T, Voss JG. Simulated flight, muscle genetics, and inflammatory indicators in mice. Aviat Space Environ Med. 2013; 84(8):840-844.

- Dobek GL, Fulkerson ND, Nicholas J, Schneider BS. Mouse model of muscle crush injury of the legs. Comp Med. 2013; 63(3):227-232.

- Nicholas J, Voss JG, Tsuji J, Fulkerson ND, Soulakova J, Schneider BS. Time course of chemokine expression and leukocyte infiltration after acute skeletal muscle injury in mice. Innate Immun. 2015; 21(3):266-274.

These articles are submitted separately.

The following sections of this report address research questions 3, 4, and 5.

3.0 M13A

Estrogen or estradiol attenuates the inflammatory response to tissue injury. For example, in a rat trauma-hemorrhage model, estrogen attenuated splenic macrophage interleukin (IL)-6 and tumor necrosis factor (TNF) messenger ribonucleic acid (mRNA) reduction [6]. Miller et al. found that estrogen decreased IL-1 and IL-6 mRNA levels in injured rat arteries [7]. Additionally, Enns, Iqbal, and Tiidus reported that estrogen decreased neutrophil and macrophage infiltration after muscle injury induced by downhill running [8].

Although the effect of estrogen is evident in rats [8,9], a 17-day estradiol treatment in mice did not attenuate leukocyte infiltration after muscle injury [10]. One possible explanation for this differing estrogen effect is that mice need to be treated with estrogen for a longer period. The finding that estrogen treatment of approximately 30-45 days induces lower body weight in mice [11,12] substantiates this possibility. When estrogen attenuates leukocyte infiltration in the rat, this effect is accompanied by lower body weight and blood insulin-like growth factor-1 (IGF-1) level [9]. Therefore, estrogen's attenuation of leukocyte infiltration in mice may occur when estrogen treatment concomitantly lowers body weight and blood IGF-1 as demonstrated in rats. Based on the findings of Gorzek et al. [11] and Moran et al. [12], the estrogen treatment period that is apt to result in these effects is approximately 30 days or more.

M13A Objective

To test the effect of long-term estrogen treatment on murine body weight and blood IGF-1.

M13A Research Question

Will 4 weeks of estradiol treatment induce an altered metabolic response in comparison with 2.5 weeks of estradiol treatment?

M13A Hypothesis

Long-term estrogen treatment lowers murine body weight and blood IGF-1.

3.1 Methods

3.1.1 Animals. Twenty-two female C57BL/6NHsd mice (*Mus musculus*) ovariectomized at 5 weeks were purchased from Harlan Laboratories (Indianapolis, IN) and allowed at least 8 days to acclimate before the start of the study. Animals were housed in a specific pathogen-free facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, at the University of Nevada, Las Vegas (UNLV). Mice were individually housed under a 12:12-hour light:dark cycle in static polycarbonate microisolator cages (Alternative Design, Siloam Springs, AR) on 0.25 in corn-cob bedding (Andersons/Bed-O'Cobs, Maumee, OH). Cotton nesting material was provided for enrichment (Nestlets; Ancare, Bellmore, NY). Tap water and rodent chow (Lab Diet 5001; Purina Mills Inc., St. Louis, MO) were available ad libitum. All animal procedures were reviewed and approved by the UNLV Institutional Animal Care and Use Committee (IACUC) and the U.S. Air Force (USAF) IACUC.

Each mouse was assigned to one of four groups: (a) placebo treatment (ovariectomized control [OC]) for 17 days (OC17), (b) estrogen treatment (ovariectomized estradiol [OE]) for 17 days (OE17), (c) placebo treatment for 51 days (OC51), and (d) estrogen treatment (OE51) for 51 days. Power analysis indicated that at least five mice per group were sufficient to detect significant differences in body weight and IGF-1 between estrogen and placebo groups. Two mice were removed from the study because of a hemorrhage during pellet implantation or incomplete ovariectomy. Final group sizes were OC17 (n = 5), OE17 (n = 5), OC51 (n = 4), and OE51 (n = 6). These group sizes were similar to that used by Gorzek et al. [11].

3.1.2 Pellet Implantation. At the time of pellet implantation, mice were 8-9 weeks old. Anesthesia for each mouse was induced with inhalant isoflurane (4%) and supplemental 100% oxygen in an induction chamber and maintained with isoflurane anesthesia and oxygen supplementation through a nose cone. The fur of the dorsal neck and back was shaved, and the skin was prepped using povidone-iodine and 70% isopropyl alcohol swabs. The skin of the neck was tented, and a sterile, 10-gauge, stainless steel trochar (Innovative Research of America, Sarasota, FL) containing a sterile, 0.18-mg 17-β-estradiol or placebo pellet (Innovative Research of America) was inserted through the skin into the subcutaneous space. This estradiol pellet was used because Gorzek et al. [11] and Moran et al. [12] reported that ovariectomized mice receiving this pellet had a 9-22% lower body weight than ovariectomized mice receiving the placebo pellet after about 30-45 days. The estradiol pellet, 0.18 mg, delivers approximately 3.0 μg of estradiol per day for up to 60 days. Gorzek et al. [11] reported that this dosage yields a mean blood estradiol level of 47 pg/mL. The incision was closed with cyanoacrylate surgical adhesive (Vetclose; Butler Schein, Dublin, OH).

Mice were allowed to recover on a heated water recirculating blanket (T/Pump; Gaymar Industries, Orchard Park, NY) and then housed in the animal care facility for 17 or 51 days post-implantation. Mice were weighed at least once per week.

3.1.3 Tissue Harvest and Euthanasia. At 17 or 51 days post-implantation, mice were anesthetized with inhalant isoflurane (3-4%) and supplemental 100% oxygen and the hind limbs were shaved. The plantarflexor muscles (soleus [n = 21], plantaris [n = 21], and gastrocnemius [n = 21]) and quadriceps muscles (n = 11) were collected for future analysis if needed. After collecting, the muscles were mounted on cork, coated with freezing medium, and then immersed in melting isopentane that was cooled by liquid nitrogen. All frozen muscles were stored at -70°C.

Blood was also collected (n = 21) prior to euthanasia for serum IGF-1 level analysis. The blood was immediately stored at 4°C, allowed to clot, and centrifuged (Eppendorf 5417c, Hamburg, Germany) for 10 minutes at 1882 X g, and then the serum was collected and stored at -70°C.

After blood collection, mice were euthanized under anesthesia by cervical dislocation, and then the uterine horns (UHs) (n = 21) were removed and weighed to determine estrogen treatment responsiveness or confirm ovariectomy.

3.1.4 Serum IGF-1 Level. A commercial IGF-1 enzyme-linked immunosorbent assay (ELISA) kit (Quantikine Mouse/Rat IGF-1; R&D Systems, Minneapolis, MN) was used to measure the serum IGF-1 level. Serum was thawed and diluted at 1:1000 with sample diluent included in the kit. Samples were run in duplicate in a 96-well plate with the provided control and standard

samples. Results were read using a plate reader (Spectramax 340PC; Molecular Devices, Sunnyvale, CA) and SoftMax Pro software (Molecular Devices). The standard curve R^2 was .996.

3.1.5 Statistical Analysis. Three study hypotheses were tested.

- Hypothesis #1. The body weight and UH weights of ovariectomized mice treated with estrogen for 17 days are higher than that of ovariectomized mice treated with placebo for 17 days.
- Hypothesis #2. The serum IGF-1 level of ovariectomized mice treated with estrogen or placebo for 17 days is similar.
- Hypothesis #3. The body weight and serum IGF-1 level of ovariectomized mice treated with estrogen are lower than that of ovariectomized mice treated with placebo for 51 days.

Statistical analyses were performed using SAS version 9.2 (SAS Institute, Inc., Cary, NC) and SPSS version 19 (IBM, Armonk, NY). The exact Wilcoxon score test was used to test all hypotheses, and Pearson's correlation was used to examine the relations between euthanasia body weight and serum IGF-1 level. Alpha was set at .05. Means ± standard error (SE) and one-sided *p*-values are reported for the hypotheses tests while the two-sided *p*-value is reported for the correlation test.

3.2 Results

3.2.1 Mouse Body Weights and UH Weights. Table 1 shows body weight (BW) and absolute and relative UH weight. Before pellet implantation, BW was similar between OC17 and OE17 ($p = .50$) and OC51 and OE51 ($p = .17$) groups. At euthanasia, the BW of the OE17 group was significantly higher than that of the OC17 group ($p = .04$). No significant difference in the euthanasia BW between the OC51 and OE51 groups was detected ($p = .08$). The absolute and relative UH weights of OE17 and OE51 mice were higher than that of OC17 ($p = .004$) and OC51 ($p = .005$) mice, respectively.

Table 1. Body and UH Weights

Group	BW1[a] (g)	BW2[a] (g)	UH (g)	UH/BW2 (mg/g)
OC17 ($n = 5$)	21.38 ± 1.15	21.80 ± 0.98	0.02 ± .002	0.85 ± 0.14
OE17 ($n = 5$)	21.80 ± 0.47	23.66 ± 0.54	0.12 ± 0.01	5.02 ± 0.34
OC51 ($n = 4$)	22.73 ± 0.89	27.38 ± 1.45	0.03 ± 0.02	1.34 ± 0.78
OE51 ($n = 6$)	21.35 ± 0.74	24.35 ± 0.53	0.16 ± 0.01	6.49 ± 0.55

[a]BW1 = body weight precrush injury or pellet implantation;
BW2 = body weight prechamber or at euthanasia.

3.2.2 Serum IGF-1 Level. The serum IGF-1 level of OE17 mice was 31% higher than that of OC17 mice (p = .05, Figure 1). There was also no significant difference in serum IGF-1 level between OC51 and OE51 mice (p = .46).

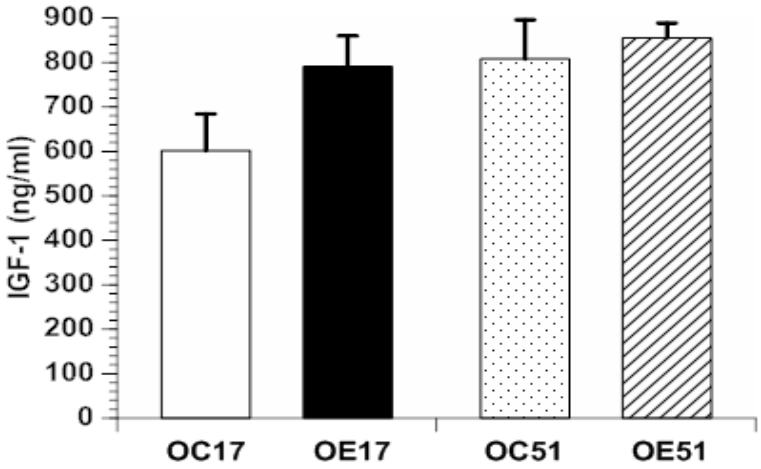


Figure 1. Serum IGF-1 level at day 17 or 51 of estrogen or placebo treatment.

3.3 Discussion

Our finding of no statistical difference in BW between OC51 and OE51 mice (hypothesis #3) is unexpected and differs from previous studies in which the mice were treated with the same estrogen dose and for a similar amount of time [11,12]. One major difference between the mice of the current and previous study is age. The mice in the current study were peripubertal (5 weeks old) at the time of ovariectomy, whereas the mice in the previous studies were post-pubertal (14-16 weeks old) [11,12]. However, a link between OC and OE mice BW difference and age at ovariectomy was not a focus of previous studies. Further investigation is needed to confirm this link to support this explanation.

Another factor difference between the current data and that of Gorzek et al. [11] is physical activity. In the Gorzek et al. study, the mice were given free access to a running wheel, and the OE mice exhibited a significantly higher distance run per 24 hours than the OC mice. Because the OC and OE BW difference was statistically evident 1 week after the OE mice demonstrated this higher distance run, Gorzek et al. speculated that the increased physical activity influenced BW gain. Additionally, the increased physical activity might have sensitized the OE mice to less weight gain because Gorzek et al. detected a significant difference between OE and OC mice with a small sample size. Therefore, a combination of long-term estrogen treatment and increased physical activity may readily prevent weight gain in OE mice.

Our finding that blood IGF-1 was not statistically different between OC51 and OE51 (hypothesis #3) is consistent with the lack of a significant BW difference between the two groups. Previous data indicate a significant, positive correlation (r = .67) between blood IGF-1 and BW in ovariectomized rats [13]. When all our IGF-1 and BW data are pooled, we detect a significant, positive correlation (r = .52, p = .02). However, this relation is not significant when the OC51 and OE51 are analyzed together, suggesting that our group size must be larger to

examine this relation between blood IGF-1 and BW of mice treated with placebo or estrogen for 51 days. Additionally, a statistical difference in blood IGF-1 of OC17 and OE17 might have been detected with a larger group size (hypothesis #2) because the *p*-value with five mice per group approached significance (.05).

In contrast to the OC51 and OE51 euthanasia BW findings, the euthanasia BW of OE17 was statistically higher than that of OC17 (hypothesis #1). Although Schneider et al. detected an OE euthanasia BW higher than that of OC after a similar estrogen and placebo treatment period, the OE BW was not statistically significant from that of the OC group [10]. The higher estradiol dosage of the current study may account for this difference. However, the relative UH weight of OE17 in the current study is slightly lower [10] or higher [14] than data of previous studies in which a lower estradiol dosage was used. Because the two previous studies were conducted in a different location than the current study, other factors unrelated to estradiol dosage may explain this statistical difference of the current study.

Based on the current study's results, two major conclusions can be drawn. One conclusion is that the duration of estrogen treatment affects the BW response of mice ovariectomized at the age of puberty. Short-term treatment increases BW whereas long-term treatment is likely to reduce BW. Additionally, the effect of long-term estrogen treatment on preventing weight gain is more apparent when administered to mice ovariectomized post-puberty and mice that engage in increased physical activity.

A second conclusion is that the examination of an estrogen effect on BW and blood IGF-1 level in ovariectomized C57BL/6 mice without increased physical activity requires more than five mice per group. Based on the group sizes of Moran et al. [12] and Fisher et al. [13], at least eight mice per group are needed to detect the effect of estrogen on BW and blood IGF-1.

4.0 M11

A potential challenge of aeromedical evacuation, also known as en route care, is the resultant mild hypobaria. Typically, the cabin of fixed-wing aircraft used for en route care is pressurized to an altitude of ~8,000 feet [2]. At this altitude, the blood oxygen saturation of a healthy person declines to 8-10% below normal [2], and the oxygen saturation of wounded soldiers drops even lower [3]. Therefore, the mild hypobaric (HB) conditions encountered during en route care may result in a reduced oxygen supply for injured and uninjured tissues.

The effect of mild HB exposure for 8-9 hours on uninjured and injured skeletal muscle is a new area of inquiry within en route care research. Recently, St. Pierre Schneider et al. showed that mild HB exposure for ~8-9 hours did not induce neutrophil and macrophage infiltration or increase pro-flammatory cytokine mRNA in uninjured muscle [15]. In addition, Ritenour et al. found no difference in the amount of muscle edema, histology, oxidant stress, or myeloperoxidase activity between rats exposed to normobaria and hypobaria (552 torr, 10,000 feet) for 5 hours immediately after skeletal muscle ischemia/reperfusion injury [16]. However, the 2-hour time period of ischemia may have caused only minimal injury, hindering the detection of a significant difference between the NB and HB groups [16]. Therefore, the acute HH effect requires more investigation.

M11 Objective

To examine whether acute HH exposure will affect neutrophil and macrophage infiltration and alter gene expression in lower extremity crush-injured muscle of mice.

M11 Research Question

Will acute HH exposure enhance neutrophil and macrophage infiltration and pro-inflammatory cytokine production and alter gene expression in lower extremity crush-injured muscle of mice?

M11 Hypothesis

Acute HH exposure enhances neutrophil and macrophage infiltration and pro-inflammatory cytokine production and alters gene expression in lower extremity crush-injured muscle of mice.

4.1 Methods

4.1.1 Animals. In total, 154 gonadal-intact female C57BL/6NHsd mice (Harlan Laboratories, Indianapolis, IN), 7-12 weeks of age, were used in this study. For the immunohistochemistry and microarray analyses, 59 mice were assigned to one of two groups: crush injury + normobaria (CINB; n = 18 and n = 12, respectively) and crush injury + hypobaria (CIHB; n = 17 and n = 12, respectively). Within each group, mice were euthanized at 32 hours or 48 hours per crush. For the flow cytometry analysis, 95 mice were assigned to one of four groups: CINB (n = 24); CIHB (n = 24); sham + normobaria (SHNB; n = 23); and sham + hypobaria (SHHB; n = 24). Within each group, mice were euthanized at 32, 96, and 192 hours. Ninety-nine animals were individually housed upon arrival. Due to limited housing space, 55 animals were group housed until crush injury, then individually housed. All mice were housed in a specific pathogen-free facility, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, at UNLV. Mice were kept in a 12:12-hour light:dark cycle, and food and water were available ad libitum. Animal procedures were reviewed and approved by UNLV and the USAF IACUCs. All mice were allowed at least 5 days to acclimate before the start of the study.

4.1.2 Crush Muscle Injury and Post-Crush Pressure Exposure. Prior to crush injury, each mouse was administered buprenorphine (Buprenex; Reckitt Benckiser Pharmaceuticals, Richmond, VA), 0.05-0.10 mg/kg subcutaneously. After 20 minutes, the mice were anesthetized with inhalation isoflurane (1-5% to effect) and 100% oxygen and then subjected to unilateral lower extremity crush muscle injury similar to a previous experiment [17]. The gastrocnemius muscle was targeted in mice used for immunohistochemistry and microarray analyses. Although gastrocnemius and quadriceps muscles were targeted in mice used for flow cytometry, only the gastrocnemius muscles underwent flow cytometry analysis. Sham mice received buprenorphine at the same dosage but did not undergo crush muscle injury. Each mouse was assessed twice daily for signs of unrelieved pain and abnormal gait or posture, and additional doses of buprenorphine were administered every 8-14 hours. No mouse received more than three doses of buprenorphine.

At 20-24 hours post-crush, each mouse was weighed, then placed—unanesthetized and individually—in a static cage containing food, water, corn-cob bedding, and nesting material. This cage was then placed in a clear, plastic rectangular container (SHNB and CINB used for flow cytometry) or in a cylindrical chamber (other mice, including CINB used for microarray and immunolabeling) as described previously [15]. The atmospheric pressure within the chamber for SHHB and CIHB mice was lowered to that equivalent to 8,000 feet by an attached vacuum

pump. The pressure inside the chamber or container in which CINB were placed was not changed. After 8-9 hours, the mice were weighed and were either returned to their cages or immediately euthanized for the 32-hour time point. Mice returned to cages were euthanized at 48, 96, or 192 hours post-crush. Immediately before euthanasia, mice were anesthetized with inhalant isoflurane (3-4%) and supplemental 100% oxygen, and blood was collected via axillary vessels or cardiac puncture. Then, mice were euthanized under anesthesia by cervical dislocation. Tissues were collected.

At the time of euthanasia, mice were anesthetized with inhalant isoflurane (3-4%) and supplemental 100% oxygen and the hind limbs were shaved. Aliquots of whole blood were collected for flow cytometry.

After blood collection, mice were euthanized under anesthesia by cervical dislocation. For flow cytometry, the gastrocnemius muscle was harvested and prepared into a single-cell suspension using gentleMACS equipment (Miltenyi Biotec, Auburn, CA) and the manufacturer's Skeletal Muscle Dissociation Kit protocol. For the microarray and mRNA analyses, the lateral gastrocnemius muscle was separated from the other muscles, flash frozen in liquid nitrogen, and stored at -150°C. For general morphological and/or leukocyte analysis, plantarflexor muscles were mounted on cork using freezing medium (Triangle Biomedical Sciences Inc., Durham, NC), immersed in melting isopentane cooled by liquid nitrogen, and stored at -70°C.

Following plantarflexor muscle harvest, the bone marrow and spleen were harvested in 95 mice. Cells were isolated from these tissues using standard procedures.

In 59 mice, the lungs were harvested after collecting the plantarflexor muscles. To prepare the lungs for freezing, the trachea was incised, and a blunt cannula attached to a 1-mL syringe containing tissue-freezing medium (Triangle Biomedical Sciences Inc.) was inserted into the proximal trachea. The tissue-freezing medium was slowly instilled through the trachea into the lungs using caution to avoid hyperinflation. After the tissue-freezing medium instillation, the lungs and trachea were removed en bloc, and the left lobe was dissected, mounted on cork using freezing medium, frozen in melting isopentane cooled by liquid nitrogen, and then stored at -70°C. The right lobe was dissected and frozen in direct liquid nitrogen and then stored at -150°C.

The UHs of all mice were removed and weighed. This tissue was used to determine the ovarian hormone status of the female mice.

The procedures involving the 95 mice represented a collaboration with Dr. Charles Caldwell and his research team, University of Cincinnati, and required this team to conduct its analyses at UNLV within 1 week. Therefore, the study was designed to accommodate this logistic and was conducted on two separate occasions. As possible, mice from all groups were tested during these two separate occasions.

4.1.3 Preparation of Muscle and Lung Cross-Sections for Leukocyte Analysis. Muscle and lung sections were cut using a cryostat (Leica CM1850; Leica Microsystems Inc., Bannockburn, IL). Ten-micron-thick frozen cross-sections were cut from the muscle area containing a hematoma. These cross-sections were applied to poly-L-lysine-coated slides and stored at -70°C until immunoperoxidase labeling. Muscle sections were also stained with hematoxylin and eosin (n = 59) to examine general morphology. Five-micron-thick frozen longitudinal sections were cut from the left lung, applied to poly-L-lysine-coated slides, and stored at -70°C until fluorescent immunolabeling.

4.1.4 Muscle Leukocyte Analysis by Immunolabeling and Computer-Assisted Image Analysis.

4.1.4.1 Immunolabeling. At 32 hours post-crush, cross-sections from 11-12 NB and 10-11 HB mice were immunolabeled for analysis. The primary monoclonal antibodies and the immunolabeling procedure were the same as used in M8 and M9 [15].

4.1.4.2 Image Analysis. The equipment and software used in this milestone were the same as those used in M9 [17]. Images of 7/4, Gr-1, 1A8, CD68, and F4/80 antigen immunolabeling were captured from an area with the most immunolabeling within the lateral gastrocnemius muscle. Each image represented an area of interest (AOI), and one AOI (0.103 mm^2) was generated for each mouse. Using the microscope, a first observer checked the AOI for obvious debris, which was marked on an image printout. Due to the size of the AOI, extraneous muscle features such as blood vessels, connective tissue spacing, and nerve bundles were not erased but avoided, if possible. A second observer (a) confirmed that the AOI was in the area with the most immunolabeling and (b) checked for debris in the AOI. Any debris was then erased manually. A second observer double-checked this erasing. An adjusted AOI was created from each erased image. The range of the adjusted AOIs was 0.099-0.103 mm^2 for each antibody.

Antibody images were converted to 8-bit gray scale, and minimum and maximum pixel limits were defined. A threshold value was then determined by assigning pixels to objects within the AOI based on the overall staining pattern observed on the computer screen. The first observer noted the reasons for the chosen threshold value (e.g., threshold value was chosen to avoid cell edges or rough tissue) on an image printout. The second observer independently selected a threshold value based on the first observer's reasons. Both observers assessed whether the selected threshold value was representative of the overall immunolabeling as viewed on the computer screen. The final threshold was then determined based on the first and second observers' values. If the observers' threshold values yielded total pixel amounts within 15% of each other, a final threshold value was selected. If the two observers' threshold values differed by 1, the highest threshold value was selected; if the values differed by 2, the middle value was selected. If the first and second observers' threshold values (a) yielded total pixel amounts that were not within 15% of each other and/or (b) differed by more than 2, the first and second observers discussed the discrepancy, and the final threshold value was arrived by consensus. Seven data points (sum, samples, min, max, range, mean, and standard deviation) obtained from the final threshold value were then exported to Microsoft Excel 2010. For each adjusted AOI per antibody, three measures were completed: number, area percentage, and mean antigen area. Number is the number of antibody-positive objects identified by the Image-Pro® Plus (known as samples) at the selected final threshold value. The area percentage is the percentage of area of antibody-positive objects. To calculate this percentage per adjusted AOI, (a) pixels were converted to area (μm^2), (b) this area was divided by the adjusted AOI area, and then (c) the quotient was multiplied by 100. The mean antigen area is the mean size of the antibody-positive objects. This mean was generated by the Image-Pro® Plus software.

4.1.5 Lung Immunolabeling. Lung sections were fixed in room-temperature acetone, rinsed in phosphate-buffered saline (PBS), and then blocked with 5% normal goat serum in PBS. The primary antibodies, rat anti-mouse Ly-6G (1A8, 1:20; BD Biosciences Pharmingen), rat anti-mouse CD68 (1:100; AbD Serotec), and rat anti-mouse CD11b (1:20; AbD Serotec), were applied to separate sections for 2 hours. After a PBS rinse, the sections were incubated for

30 minutes with the secondary antibody, Alexa Fluor® 488 goat anti-rat IgG (1:800; Invitrogen, Carlsbad, CA). After a PBS and a water wash, the sections were mounted with an aqueous medium (Slow Fade® Gold Anti-Fade Reagent with diamidino phenylindole; Invitrogen) and coverslipped. Control sections were generated as described above, except the sections were incubated in PBS for 2 hours instead of a primary antibody. Slides were visualized using an Eclipse E600 microscope (Nikon Inc., Melville, NY) with fluorescein isothiocyanate and diamidino phenylindole filters.

4.1.6 Microarray and Polymerase Chain Reaction (PCR). The microarray and PCR procedures were performed similarly as described [15]. For microarray, the cutoff threshold for gene changes was ≥ |1.5| log-fold change, and the unadjusted *p*-value was used. PCR was used to examine mRNA relative quantification levels of F4/80 antigen, CD68 antigen, IL-1β, IL-6, IL-10, and TNF.

4.1.7 Blood, Bone Marrow, Spleen, and Muscle Leukocyte Analysis by Flow Cytometry. The following flow cytometric-suitable antibodies were utilized: Lineage panel (catalog number 133302, BioLegend, San Diego, CA), Sca-1 (clone: D7, BioLegend), CD117 (clone: 2B8, BioLegend), CD3 (clone: 145-2C11, BD Biosciences, San Jose, CA), B220 (clone: RA3-6B2, BD Biosciences), Ly6G (clone: 1A8, BD Biosciences), Ly-6C (clone: HK1.4, BioLegend), β-TCR (clone: H57-597, BD Biosciences), CD4 (clone: RM4-5, BD Biosciences), CD8 (clone: 53-6.7, BD Biosciences), F4/80 (clone: CI:A3-1, AbD Serotec, Raleigh, NC), 7/4 (clone: 7/4, AbD Serotec), CD68 (clone: FA-11, AbD Serotec), CD11b (clone: M1/70, BD Biosciences), RORγt (clone: Q31-378, BD Biosciences), and T-Bet (clone: 4B10, BD Biosciences). Nonspecific binding to cells was controlled by adding 5% rat serum (Invitrogen, Carlsbad, CA) and 1 µg/tube of Fc Block (BD Biosciences) to the fluorescence-activated cell sorting (FACS) buffer.

Cells from the spleen, blood, and bone marrow were isolated per standard procedures. Splenocytes were analyzed using four panels of antibodies that recognize the following antigens: (a) CD4, CD8, CD3, and B220; (b) intracellular T-Bet and intracellular RORγt; (c) F4/80, 7/4, and intracellular CD68; and (d) Ly-6G, Ly-6C, and CD11b. Peripheral blood cells were analyzed using antibodies that recognize Ly-6G, Ly-6C, CD3, and B220. Bone marrow cells were analyzed using two antibody panels: (a) Lineage panel, CD117, and Sca-1 and (b) Ly-6G, Ly-6C, CD3, and B220. To evaluate CD68, RORγt, or T-Bet *in situ*, intracellular staining was conducted using paraformaldehyde-fixed—then permeabilized—cells using 0.1% saponin in PBS; the cells were then stored at -20°C until analysis. After antibody labeling, cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and Cell Quest software (BD Biosciences).

Single-cell suspensions were obtained from the gastrocnemius muscle using gentleMACS equipment (Miltenyi Biotec, Auburn, CA) and the manufacturer's Skeletal Muscle Dissociation Kit protocol. Furthermore, cells were counted with a Scepter 2.0 per the manufacturer's instructions (EMD Millipore, Billerica, MA), then labeled with an antibody that recognizes intracellular CD68, F4/80 antibody, 7/4 antibody, and/or anti-Ly-6G, anti-Ly-6C, or anti-CD11b antibodies. Cells with one of four distinct antigen patterns were of interest: (a) positive for the CD68 and F4/80 antigens and a low level of the 7/4 antigen, (b) positive for CD68 and 7/4 antigens and a low level of the F4/80 antigen, (c) positive for Ly-6G and Ly-6C antigens, and (d) positive for Ly-6C and a low level of Ly-6G.

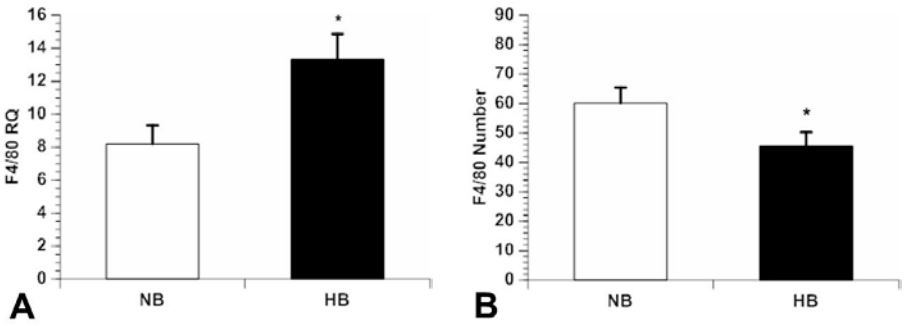
4.1.8 Statistical Analysis. Data were analyzed using SAS versions 9.2 and 9.3 and SPSS version 16. The Wilcoxon two-sample test was used to compare the difference in mRNA levels and leukocyte antigen number, area percentage, and mean antigen area between the NB and HB groups. Alpha was set at .05 for these analyses.

Flow cytometry data statistical analysis of blood, bone marrow myeloid cells, spleen myeloid cells, spleen T and B cells, and muscle myeloid cells was initiated by comparing the four groups, SHNB, SHHB, CINB, and CIHB, using the Kruskal-Wallis test. A comparison of CINB versus CIHB at 32 and 96 hours was performed using a generalized fixed sequence (or gatekeeping) method to adjust for multiple comparisons. If resulting adjusted *p*-values were $< .05$, the comparisons were declared significant.

The microarray data were analyzed as previously described (see Appendix). Probesets that were differentially expressed at a $\geq |1.5|$-fold level and had an unadjusted $p < .05$ were considered significant and reported in Results.

4.2 Results

4.2.1 F4/80 mRNA and F4/80-Positive Cells in Crush-Injured Muscle. The F4/80 mRNA level of the HB group was significantly higher than that of the NB group (Figure 2). Figure 2 also shows that fewer F4/80-positive cells were present in crush-injured muscle of HB mice than NB mice at 32 hours post-crush. The difference in F4/80 area percentage between HB and NB mice approached significance ($p = .06$). Despite the visual observation of fewer F4/80-positive cells in crush-injured muscle of HB mice than NB mice at 48 hours post-crush, no significant difference in F4/80 number, F4/80 area percentage, and mean F4/80 antigen area was found.



4.2.2 Muscle Myeloid Cell Phenotypes by Flow Cytometry. To compare flow cytometry findings between CINB and CIHB at 32 and 96 hours post-crush, a generalized fixed sequence method was used. Among the eight variables, only one, CD68+F4/80+7/4lo, yielded a significant difference at 32 hours, adjusted *p*-value = .0302. The median number of these cells was 0.268 for CINB and 0.099 for CIHB. The variable, Ly-6G hi and Ly-6C hi, approached significance at 32 hours, adjusted *p*-value = .0714.

4.2.3 Crush-Injured Muscle Microarray Analysis. Table 2 lists the gene expression changes at 32 and 48 hours post-crush between CINB and CIHB groups. At 32 hours post-crush, 10 genes were upregulated and 9 genes were downregulated in the CIHB group. At 48 hours post-crush,

0 genes were upregulated and 9 genes were downregulated. The downregulated genes at 32 and 48 hours post-crush were different. Of the upregulated genes, a specific gene associated with leukocytes is CXCL14, which is a chemokine that will attract monocytes and dendritic cells. Among the downregulated genes at 48 hours, notable genes associated with leukocytes are ARG-1 and SNLF4. ARG-1 is found in alternatively activated macrophages. SNLF4 downregulation occurs when macrophages are undergoing differentiation [18].

Table 2. HB-Upregulated and Downregulated Genes at 32 and 48 Hours Post-Crush

Gene Symbol		Fold Change	*p*-value
HB-Upregulated Genes at 32 Hours Post-Crush			
Prg4	Proteoglycan 4	1.51	.0218
Zfp947	Zinc finger protein 947	1.51	.0034
Npas2	Neuronal PAS domain protein 2	1.56	.0026
Trim12c	Tripartite motif-containing 12C	1.56	.0056
Mcpt4	Mast cell protease 4, isoform CRA	1.64	.0006
Cxcl14	C-X-C motif chemokine 14	1.66	.0019
Gm4841	Predicted gene 4841	1.69	.0070
Fbxl22	F-box and leucine-rich repeat protein 22	1.70	.0022
Serpinb1a	Leukocyte elastase inhibitor A	1.75	.0127
Cpa3	Mast cell carboxypeptidase A	1.80	.0038
HB-Downregulated Genes at 32 Hours Post-Crush			
BB287469	No description available	1.53	.0127
Mpz	Myelin protein zero	1.54	.0049
Cish	Cytokine-inducible SH2-containing protein	1.54	.0032
Olfr171	Olfactory receptor 171	1.55	.0078
Snord116	Small nucleolar RNA, C/D box 116 cluster	1.58	.0081
Timp4	Metalloproteinase inhibitor 4	1.61	.0053
AI747448	No description available	1.65	.0002
Mrgpra9	MAS-related GPR, member A9	1.65	.0160
Cyp2e1	Cytochrome P450, family 2, subfamily E, polypeptide 1	1.93	.0104
HB-Downregulated Genes at 48 Hours Post-Crush			
IL6	Interleukin-6	1.52	.0057
AI504432	No description available	1.52	.0173
Phf11	PHD finger protein 11	1.59	.0017
Pyhin1	Pyrin and HIN domain family, member 1	1.60	.0109
Plac8	Placenta-specific gene 8 protein	1.66	.0011
Arg1	Arginase-1	1.77	.0422
Pydc4	Pyrin domain containing 4	1.77	.0011
Ms4a4c	Membrane-spanning 4-domains, subfamily A, member 4C	1.97	.0073
Snlf4	Schlafen 4	2.16	.0021

4.2.4 Other Leukocyte Antigens and Other Tissues. Significant differences with the other leukocyte antigens in muscle between CINB and CIHB were not detected by PCR, immunohistochemistry, or flow cytometry. Also, no significant differences with the other leukocyte antigens in other tissues between CINB and CIHB were detected by flow cytometry or immunohistochemistry.

4.3 Discussion

In this study, exposure to hypobaria, starting approximately 24 hours post-crush and ending at 32 hours post-crush, induced two major changes in crush-injured muscle. One change is the downregulation of macrophage-related genes at 48 hours post-crush. A second change is the reduction in the number of F4/80-positive leukocytes at 32 hours post-crush and elevation of the F4/80 mRNA level at 32 hours post-crush. In contrast, hypobaria had no unique effect on other leukocytes in the crush-injured muscle or in other tissues that are leukocyte sources for injured muscle (e.g., bone marrow and blood). Therefore, hypobaria appears to directly affect cellular and gene changes within the crush-injured muscle. This study is the first investigation to report these macrophage-related changes associated with acute mild hypobaria

We hypothesized that acute HH exposure would alter gene expression in lower extremity crush-injured muscle. Through microarray analysis, this hypothesis was supported. Specifically, genes were upregulated and downregulated at 32 hours post-crush and only downregulated at 48 hours post-crush. Several of the downregulated genes are associated with macrophages. Further research is needed to determine whether protein expression is affected.

Because of the lack of extant data regarding acute mild HH effects on injured muscle, the study's hypothesis regarding the enhancement of neutrophil and macrophage infiltration was based on data generated from short-term, severe HH studies involving cells in culture or uninjured brain. These extant data are conflicting. For example, SaiRam et al. demonstrated enhanced macrophage phagocytosis after a 2-hour exposure to hypobaria (~25,000 feet) [19]. Klokker et al. reported that a 20-minute exposure to ~18,000 feet increased neutrophil oxidative activities 2 hours after hypobaria [20]. In contrast, Hitomi et al. found that the neutrophil oxidative activity, superoxide anion generation, was unaffected by a 2-hour exposure to hypobaria (~15,000 feet) [21]. Additionally, Al-Saleh, Kaur, and Ling showed that short-term hypobaria (~26,000 feet) led to an immediate decrease in antigens expressed by brain macrophages, but later (7-14 days) macrophage antigen upregulation occurred [22]. Based on these previous findings, we hypothesized that acute mild hypobaria would enhance neutrophil and macrophage infiltration. Our findings suggest that our hypothesis was not supported. Instead, acute mild hypobaria appears to have no effect on neutrophil infiltration into crush-injured muscle, but has a temporary suppressive effect on the F4/80 subpopulation of macrophages within crush-injured muscle. Future research is needed to determine whether an extended HH exposure, such as 12-16 hours, would suppress F4/80-positive macrophage infiltration at post-crush time points beyond 32 hours. If this extended HH exposure, which is relevant for the en route care in the Pacific theater, were to suppress F4/80-positive macrophage infiltration beyond 32 hours post-crush, then muscle regeneration might be hindered. The first 48 hours post-crush are critical for removing injured tissue so that muscle regeneration can proceed in a timely manner.

5.0 M13/M15/M17

En route care, or flying wounded soldiers from the battlefield to a regional medical center 24 hours post-injury, contributes to the 91-99% survival rate of combat casualties [5]. However, wounded soldiers without supplemental oxygen are likely to experience at least one hypoxemic event during en route care [23], and one or more hypoxemic effects could hinder tissue recovery. Multiple factors may account for this event, including the aircraft cabin's HB condition.

Given the survival rate for which en route care yields, the overall impact of the HB condition and other factors may vary. For a certain subpopulation of patients, the impact may warrant attention because their survival rate is not 100% and their quality of life post-recovery may not be optimal. Therefore, strategies to counteract the aircraft cabin's HB condition need to be considered, especially in planning for 12- to 16-hour flights from the Pacific theater.

One strategic option to address hypobaria's potential negative effect on tissue recovery is to determine the "safe" time to fly: the time at which exposure to HH will have no effect on tissue recovery. However, this option might lower the survival rate because the patient, if flown before 24 hours post-injury, may fly during an unstable period after injury. The survival rate or quality of life also may be lowered because local medical centers may not have therapies to prevent certain complications if the patient is flown beyond 24 hours post-injury. Therefore, another option may need to be considered.

Another strategic option is to identify countermeasures (i.e., substances that can be administered to counteract the HB effects on tissue recovery). For this project, we tested estrogen as a countermeasure. One reason for selecting estrogen is that in a skin wound healing study, the exogenous administration of estrogen in physiological doses increased the number of alternatively activated (Ym-1-positive) macrophages at 3 and 7 days post-injury [24]. These macrophages are significant because these cells are associated with wound repair. In addition, the white blood cells that were reduced in crush-injured muscle in response to HH, F4/80-positive cells, have been identified as alternatively activated macrophages in another muscle injury model [25]. Therefore, we hypothesized that estrogen promotes the infiltration of these macrophages into the crush-injured muscle.

M13/M15/M17 Objective

To test whether estrogen will promote neutrophil and macrophage infiltration, cytokine production, and inflammation-related gene expression in lower extremity crush-injured muscle of mice exposed to acute HH.

M13/M15/M17 Research Question

Will estrogen promote neutrophil and macrophage infiltration, cytokine production, and inflammation-related gene expression in lower extremity crush-injured muscle of mice exposed to acute HH?

M13/M15/M17 Hypothesis

Estrogen promotes neutrophil and macrophage infiltration, cytokine production, and inflammation-related gene expression in lower extremity crush-injured muscle of mice exposed to acute HH.

5.1 Methods

5.1.1 Animals. In total, 163 female C57BL/6NHsd mice (Harlan Laboratories, Indianapolis, IN), 4-10 weeks of age, were used. In this milestone, 44 of these mice were gonadal-intact and 120 were ovariectomized. All mice were housed individually and in a specific pathogen-free facility, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, at UNLV. Mice were kept in a 12:12-hour light:dark cycle, and food and water were available ad libitum. Animal procedures were reviewed and approved by UNLV and the USAF IACUCs. All mice were allowed at least 5 days to acclimate before the start of the study.

5.1.2 Crush Muscle Injury and Post-Crush Pressure Exposure. Initially, the gastrocnemius and quadriceps muscles of four gonadal-intact mice underwent the crush injury procedure similar to the one previously described [17]; however, the four mice only received three doses of buprenorphine. These mice were euthanized at 96 hours post-crush to verify the presence of muscle regeneration at this time point.

The remaining gonadal-intact female mice were assigned to either the intact female (IF) 96-hour or IF 192-hour post-crush groups. The gastrocnemius and quadriceps muscles of these gonadal-intact mice underwent the crush injury procedure similar to that described in M11. Twenty to 24 hours after crush injury, these mice underwent hypobaria similar to the procedure as described in M11. One difference is that these mice only received two doses of buprenorphine with a frequency of every 8-14 hours. A third dose was only administered if the mouse exhibited signs of discomfort, pain, or difficulty in ambulating. Euthanasia was performed as described in M11. Tissue harvest included the collection of blood, plantarflexor and quadriceps muscles, and spleen. A small aliquot of whole blood was used to measure hematocrit. The remaining blood was stored on ice for at least 30 minutes and then centrifuged to separate the blood cells from the serum. The serum was used to measure circulating estradiol levels. The quadriceps muscles were frozen in liquid nitrogen and stored at -150°C to measure muscle cytokine levels. For microarray and quantitative PCR analyses, the lateral gastrocnemius was separated from the other muscles, frozen in liquid nitrogen, and stored at -150°C. For leukocyte analysis, the plantarflexor muscles were mounted on cork, coated with freezing medium, and then immersed in melting isopentane cooled by liquid nitrogen.

Ovariectomized mice were assigned to one of six groups: OC 32 hours, OC 96 hours, OC 192 hours, OE 32 hours, OE 96 hours, and OE 192 hours post-crush. Prior to the crush injury procedure, these mice underwent pellet implantation similar to the procedure described in M13A. Pellet implantation occurred at 5-10 weeks after ovariectomy. The OC mice received a 60-day release placebo pellet (0.18 mg total). The majority of the OE mice received a 60-day release 17-β estradiol pellet (0.18 mg total). However, less than five OE mice inadvertently received a 21-day release 17-β estradiol pellet (0.05 mg total). Despite the different release days of the two pellets, the amount of estradiol released per day is similar.

Seven to 8 days after pellet implantation, the gastrocnemius and quadriceps muscles of the ovariectomized mice underwent the crush injury procedure similar to the procedure as described in M11. Twenty to 24 hours after crush injury, these mice underwent hypobaria similar to the procedure as described in M11. One difference is that these mice only received two doses of buprenorphine with a frequency of every 8-14 hours. Euthanasia was performed as described in M11. Tissue harvest included the collection of blood, plantarflexor and quadriceps muscles, and spleen. A small aliquot of whole blood was used to measure hematocrit. The remaining

blood was stored on ice for at least 30 minutes and then centrifuged to separate the blood cells from the serum. The serum was used to measure circulating estradiol levels. The quadriceps muscles were frozen in liquid nitrogen, stored at -150°C, and used to measure muscle cytokine levels. For microarray and quantitative PCR analyses, the lateral gastrocnemius was separated from the other muscles, frozen in liquid nitrogen, and stored at -150°C. For leukocyte analysis, the plantarflexor muscles were mounted on cork, coated with freezing medium, and then immersed in melting isopentane cooled by liquid nitrogen.

5.1.3 Serum Estradiol Levels. To determine the amount of circulating estrogen, serum from IF, OC, and OE mice was assayed using the Mouse/Rat Estradiol ELISA kit (Calbiochem, Spring Valley, CA). The assay was performed according to the kit protocol and using a VersaMax plate reader (Molecular Devices, Sunnyvale, CA); data were analyzed with SoftmaxPro version 6 software (Molecular Devices). Samples that tested higher than the range of the standard curve were diluted 1:10 with PBS-bovine serum albumin and tested again. Also, samples with a coefficient of variance larger than 15% were also retested.

5.1.4 Muscle Cytokine Protein Levels. Uninjured and injured quadriceps muscles of 30 OC (n = 10 at 32, 96, and 192 hours post-crush), 30 OE (n = 10 at 32, 96, and 192 hours post-crush), and 18 IF (n = 8 at 96 hours and n = 10 at 192 hours post-crush) mice underwent protein extraction using a Millipore protein extraction kit (Billerica, MA), a BCA Protein Assay Kit (VWR, Visalia, CA), SoftMaxPro version 5.4 (Molecular Devices), and SPECTRAMax 340PC plate reader (Molecular Devices). Samples were stored at -150°C. To determine the amount of six cytokines in these protein-extracted samples, these samples were assayed using a Milliplex Mouse Cytokine Magnetic Bead Panel kit (Millipore, Billerica, MA) and Luminex 200^{TM} (Austin, TX). The six cytokines of interest were IL-1α, IL-1β, IL-6, eotaxin, TNF, and RANTES. The xPONENT software (Luminex) was used for data interpretation.

5.1.5 PCR and Microarray. The PCR and microarray procedures were performed similarly as described [15]. PCR was used to examine mRNA (relative quantification) levels of the muscle-specific factors MYOD, MYOG, MYF5, IGF-1, and PAX7; cytokines CXCL5, CXCL2, CXCL1, CXCR2, CCR2, CCL2, IL-1β, IL-6, IL-10, and TNF; and macrophage markers CD68, F4/80, CD206, and ARG-1.

5.1.6 Muscle Leukocyte Analysis by Immunolabeling and Computer-Assisted Image Analysis. Muscle was prepared for leukocyte analysis by immunolabeling and computer-assisted image analysis similar to what was done for M11. In addition, immunolabeling and computer-assisted image analysis procedures were performed similar to what was done for M11. One difference is that control sections were only visually inspected. Images from these sections were captured only if there was difficulty in matching the AOI in the image of the immunolabeled section with that of the control section. Also, large connective tissue regions or gaps, blood vessels, or nerve bundles were manually erased. Besides number, area percentage, and mean antigen area, the max area was examined. Max area represents the greatest area of an individual cell among all the cells within the AOI. Because the majority of muscle sections of the mice euthanized at 96 and 192 hours post-crush did not exhibit 7/4, Gr-1, and 1A8 immunolabeling, no quantification of these sections was performed.

5.1.7 Statistical Analysis. Data were analyzed using SAS version 9.2, 9.3, or 9.4. The nonparametric Kruskal-Wallis test was used to compare the serum estradiol level among groups. A two-sample, two-sided *t*-test (*p*-value for equality of variances) was performed to compare the means of injured minus uninjured at each time point for each cytokine. The PCR data were analyzed using two-sample Kruskal-Wallis test or non-parametric analysis of variance. To analyze the muscle leukocyte data, first, the overall difference was assessed using analysis of variance, and then post-hoc tests were performed using the Kruskal-Wallis procedure. Alpha was set at 0.05 and adjusted for multiple comparisons when needed.

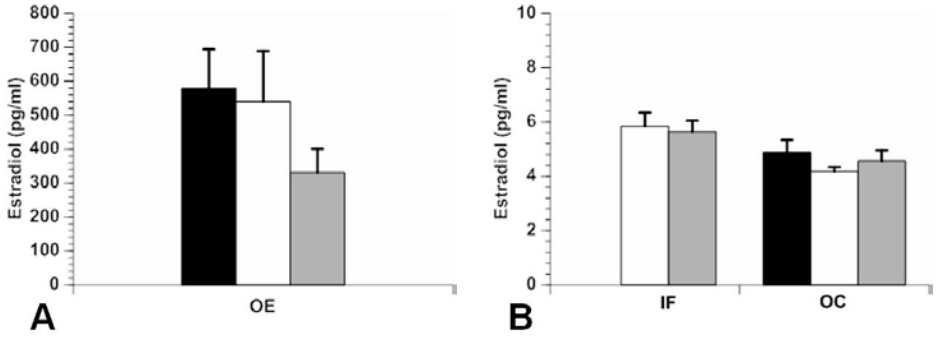
Flow cytometry data statistical analysis of blood, bone marrow myeloid cells, spleen myeloid cells, spleen T and B cells, and muscle myeloid cells was initiated by comparing the four groups, SHNB, SHHB, CINB, and CIHB, using the Kruskal-Wallis test. A comparison of CINB versus CIHB at 32 and 96 hours was performed using a generalized fixed sequence to adjust for multiple comparisons. If resulting adjusted *p*-values were < .05, the comparisons were declared significant.

The microarray data were analyzed as previously described [15]. The cutoff threshold for gene changes was $\geq$ |1.5| log-fold change, and the unadjusted or adjusted *p*-value was used. A p-value < .05 was considered to be significant.

5.2 Results

5.2.1 Serum Estradiol. The effectiveness of ovariectomy in the placebo-treated mice was verified by the presence of a relative UH weight of < 1.31 mg/g at the time of euthanasia. This weight was about six-fold lower than that of IF mice. Despite this low relative UH weight, the serum estradiol level between OC and IF mice was only significantly different at 96 hours post-crush (Figure 3).

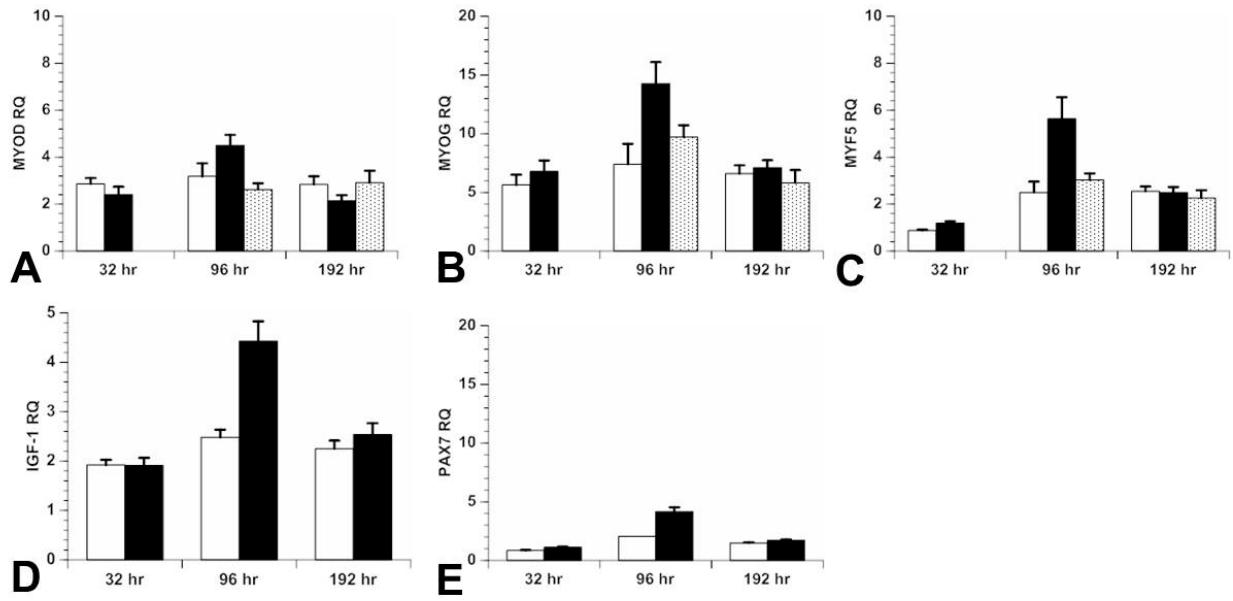
The serum estradiol level of OE mice was significantly higher than IF and OC mice at all time points ($p < .0167$, Figure 3); however, there was no significant difference in the level among the different OE groups.



5.2.2 Muscle Morphology. In this milestone, lateral gastrocnemius muscle morphology at 32 hours post-crush was similar to that of 32 hours post-crush M11. At 96 hours post-crush, all groups exhibited regenerating muscle fibers surrounded by inflammatory cells; however, at 192 hours post-crush, in most of the muscles of the IF group, remaining regenerating muscle fibers were less likely to be surrounded by inflammatory cells. These findings contrasted with those observed in the muscles of the OC group. The muscle morphology of the OC 192-hour post-crush appeared to consist of more regenerating fibers surrounded by inflammatory cells.

5.2.3 Muscle Cytokine Protein. The only significant comparison was an approximately three-fold injured-uninjured difference in RANTES between IF and OE at 192 hours post-crush. That is, the RANTES level was significantly higher in crush-injured muscle of OE than in IF mice.

5.2.4 Muscle-Specific Factor Gene Expression. The gene expression (mRNA level) of five muscle-specific factors in crush-injured muscle was examined at three post-crush time points: 32, 96, and 192 hours (Figure 4).



At 32 hours post-crush, the OE group exhibited greater MYF5 and PAX7 gene expression than the OC group. There was no significant difference in the other three genes at this time point.

At 96 hours post-crush, there was an overall significant expression in the three genes, MYOD, MYOG, and MYF5, which were compared among the OE, OC, and IF groups. Pairwise comparisons revealed a significantly higher MYOD expression in OE than in the IF mice. Also, at 96 hours post-crush, the overall expression of two genes, IGF-1 and PAX7, was significant between the OE and OC groups. Specifically, the gene expression was higher in the OE than in the OC mice.

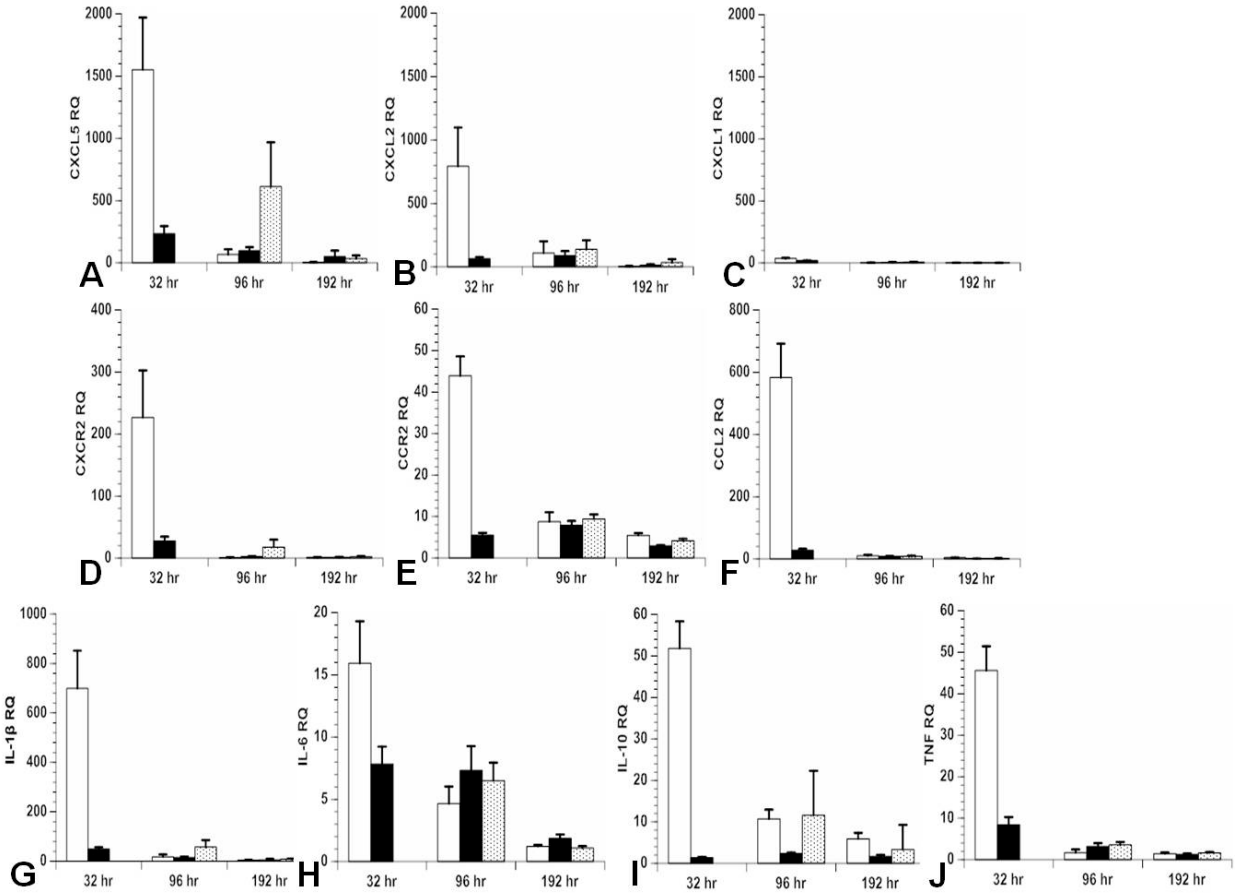
At 192 hours post-crush, no group differences were detected in any of the genes.

5.2.5 Cytokine Gene Expression. The gene expression (mRNA level) of 10 cytokines was examined (Figure 5) at three post-crush time points: 32, 96, and 192 hours.

At 32 hours post-crush, the OC group exhibited greater expression of nine of the genes than that of the OE group. IL-6 gene expression was similar between the two groups.

At 96 hours post-crush, the expression of nine genes was similar among the OE, OC, and IF groups. The exception was IL-10 gene expression. The IL-10 gene expression in the OE group was significantly lower than that of the OC and IF groups.

At 192 hours post-crush, the expression of four genes, IL-6, IL-10, CCL2, and CCR2, was significantly different among the OE, OC, and IF groups. In regard to IL-10, CCL2, and CCR2 genes, the expression of the OE group was significantly lower than that of the OC group. Cytokine differences between the OE and IF groups and OC and IF groups varied. The IL-6 expression was significantly higher in the OE group than in the IF group. In contrast, the CCR2 expression of the OE group was lower than that of the IF group. The CCL2 expression of the OC group was higher than that of the IF group.

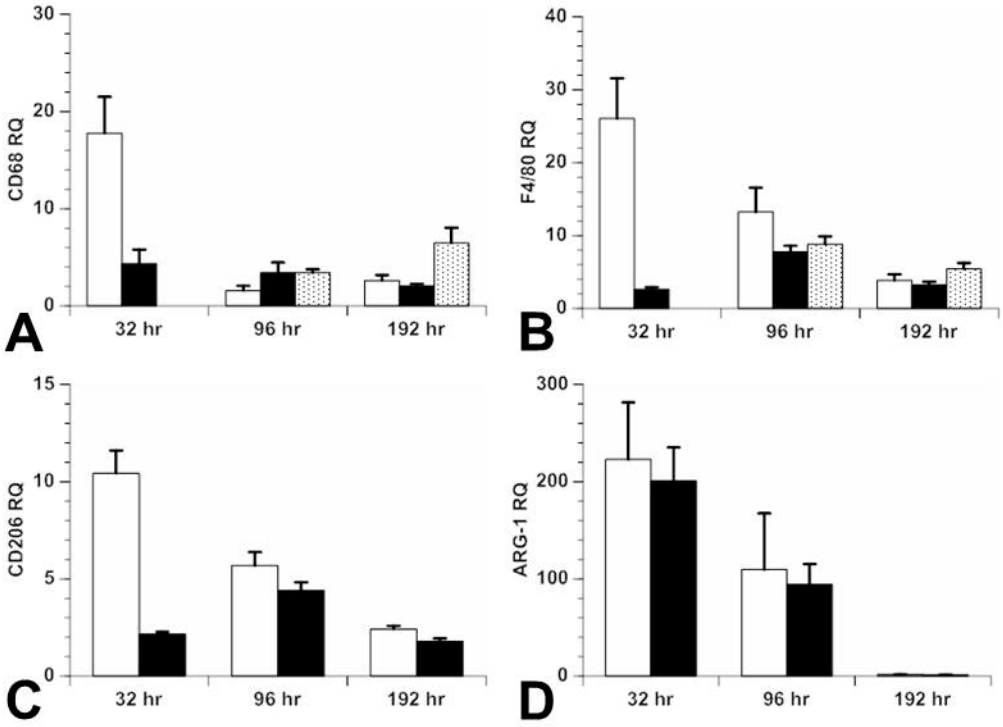


5.2.6 Macrophage Marker Gene Expression. The gene expression (mRNA level) of four macrophage markers was examined at three post-crush time points: 32, 96, and 192 hours (Figure 6).

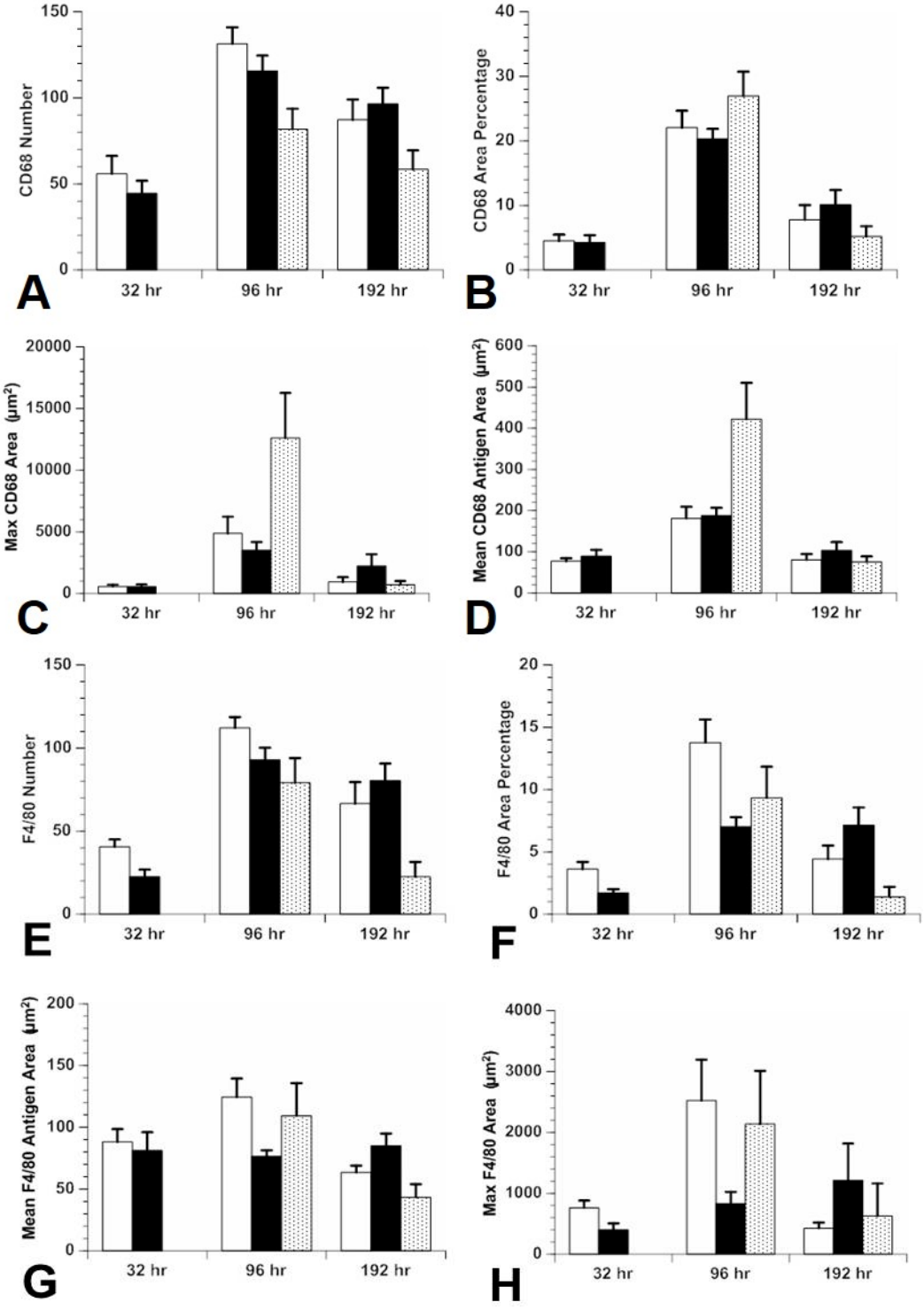
At 32 hours post-crush, the expression of three of these markers was significantly higher in the OC than in the OE group.

At 96 hours post-crush, no difference in CD68 and F4/80 gene expression was detected among the three groups, OC, OE, and IF. In addition, when CD206 and ARG-1 gene expression was compared between the OC and OE groups, no difference was detected.

At 192 hours post-crush, the expression of CD68 but not F4/80 was significantly different among the OE, OC, and IF groups. CD68 expression was greater in the IF group than in the OE group. When expression of CD206 and ARG-1 was compared between the OE and OC groups, the expression of these genes was higher in the OC group than in the OE group.



5.2.7 Macrophage and Neutrophil Infiltration. Within a designated AOI, macrophage infiltration was quantified by the number of cells, the area of percentage, mean antigen area, and max antigen area (Figure 7). At 32 and 192 hours post-crush, there was no difference in CD68-positive macrophages between the OC and OE groups and among the OC, OE, and IF groups, respectively. At 96 hours post-crush, there were significantly fewer CD68-positive macrophages in the IF group than in the OC group. At this same time point, mean CD68 area and max CD68 area were greater in the IF group than in the OE group. The CD68 area percentage was similar in all three groups at 96 hours post-crush.



When examining CD68-positive macrophages over time within treatment groups, the OC and OE groups exhibited higher values in all four variables at 96 hours post-crush than at 32 hours post-crush. The area percentage, max, and mean area values of the OC group were higher at 96 hours post-crush than at 192 hours post-crush. Only the area percentage and mean area values of the OE group were higher at 96 hours than at 192 hours post-crush. The number of CD68-positive macrophages of the OE 96-hour and 192-hour post-crush groups was similar, but the 32-hour group was significantly lower than that of the 192-hour post-crush.

F4/80-positive macrophages were higher in number and area percentage at 32 hours post-crush and higher in area percentage at 96 hours post-crush in the OC group than in the OE group. At 192 hours post-crush, F4/80-positive macrophages were higher in number and area percentage in the OC and OE groups than in the IF groups. The max F/480 area and mean F4/80 area values were similar among the groups at all time points.

When examining F4/80-positive macrophage infiltration over time within treatment groups, these macrophages were higher in number and area percentage at 96 hours post-crush than at 32 hours post-crush in both the OC and OE groups. The value of all four variables of the OC 96-hour post-crush group was higher than that of the OC 192-hour post-crush group. In contrast, number and area percentage values of the OE 32-hour post-crush group were lower than that of the OE 192-hour post-crush group. Also, max area and mean area values of all three OE groups were similar.

Within a designated AOI, neutrophil infiltration was quantified by the number of cells, the area of percentage, mean antigen area, and max antigen area at 32 hours post-crush. No differences in the values of the four variables were detected among the treatment groups.

5.2.8 Microarray Gene Expression. Three analyses were completed to examine gene expression via microarray. One analysis consisted of comparing gene expression between the OC and OE groups at all three time points. In total, 756 genes were significantly upregulated or downregulated in the OE groups in comparison with the OC groups. At 32 hours post-crush, 192 genes were upregulated and 102 genes downregulated in the OE group in comparison with the OC group. At 96 hours post-crush, 83 genes were upregulated and 69 genes downregulated in the OE group in comparison with the OC group. At 192 hours post-crush, 257 genes were upregulated and 53 genes downregulated in the OE group in comparison with the OC group. Among these changes, estrogen led to a downregulation of one of the inflammation-related signaling pathways, NFκB.

Another analysis consisted of comparing gene expression between the OE and IF groups at 96 hours post-crush and 192 hours post-crush. In total, 264 genes were significantly upregulated or downregulated in the OE groups in comparison with the IF groups. At 96 hours post-crush, 50 genes were upregulated and 57 genes were downregulated. At 192 hours post-crush, 131 genes were upregulated and 26 genes were downregulated.

The third analysis consisted of comparing gene expression between the OC and IF groups at 96 hours post-crush and 192 hours post-crush. In total, 146 genes were significantly upregulated or downregulated in the OC groups in comparison with the IF groups. At 96 hours post-crush, 20 genes were upregulated and 17 genes were downregulated. At 192 hours post-crush, 52 genes were upregulated and 57 genes were downregulated.

5.3 Discussion

There are four major and unique findings of this study. One major finding is that all neutrophil and most of the macrophage variables were similar between the OC and OE mice at 32 hours post-crush, indicating that estrogen did not counteract the HH suppressive effect at 32 hours post-crush (refer to M11). In fact, at this time point, F4/80-positive macrophage variables were lower in the OE group than in the OC group.

Another major finding is that the OC mice at 32 and 96 hours post-crush exhibited a higher level of cytokines, macrophage markers, and macrophage infiltration than the OE mice. However, by 192 hours post-crush, F4/80 macrophage infiltration was similar between the two groups, although not comparable, to that of the IF mice (see below). Also, three of the five muscle-specific factors were higher in the OE mice at 32 and/or 96 hours post-crush than in the OC mice. Collectively, these findings suggest that the completion of muscle regeneration after crush injury and HH is delayed in ovariectomized subjects without estrogen and progesterone.

Another interesting set of findings is the group changes evident at 192 hours post-crush. For example, F4/80 number and area percentage values were higher in the OC and OE groups than in the IF group. More genes were upregulated in the OE group at 192 hours post-crush than in the OC and IF groups at 32 or 96 hours post-crush. In addition, more genes were upregulated or downregulated in the OC group at 192 hours post-crush in comparison with of the IF group.

Finally, the white blood cell (both neutrophils and macrophages) response to an acute closed crush muscle injury and HH appears to be dampened in ovariectomized subjects with estrogen alone or without estrogen and progesterone in comparison with M11 IF data. For example, as shown in M11 Figure 2B, mean F4/80 number of IF groups is ~60 (CINB) and ~40 (CIHB). In M13/M15/M17, the mean F4/80 number of OC and OE 32-hour post-crush groups, which were exposed to HH, is ~40 and ~20, respectively. Therefore, the F4/80 number of all the CIHB groups is lower than that of the CINB group. While this HH effect is restricted to 32 hours post-crush, this effect may initiate a cascade of later events in the ovariectomized mice. For instance, as described above, at 192 hours post-crush, F4/80 number and area percentage values were higher in the OC and OE groups than in the IF group. Along with these changes as described above, these ovariectomized groups also have significant gene changes at 192 hours post-crush, a time at which IF (CIHB) mice demonstrated muscle recovery.

6.0 CONCLUSIONS

The overall objectives of this project were to (a) create experimental models for studying the effect of HH on muscle recovery and (b) test the effect of estrogen, as a countermeasure, on HH effects on muscle recovery. Project findings suggest that adequate experimental models were developed for studying the effect of HH on muscle recovery. The crush muscle injury model is novel in that surgical intervention is not required to target the muscle of interest. Although estrogen did not counteract the suppressive effect of HH on white blood cells, estrogen may have acted as a countermeasure for ovariectomy effects on muscle regeneration. That is, ovariectomy alone prevented significant global gene upregulation and downregulation of at least one inflammatory pathway and induced high levels of specific inflammatory-related genes. In contrast, estrogen in the ovariectomized condition had opposite effects and promoted a muscle recovery similar to that of the IF. These findings are also novel.

From a military perspective, future studies should address whether (a) the occurrence of en route care at 24 hours after an acute muscle injury affects muscle regeneration; (b) a more complex experimental model (e.g., hemodynamic changes + acute muscle injury + HH for 8-16 hours), which is more representative of the field condition, induces a greater suppressive effect on white blood cells; (c) a high estrogen dose is an effective countermeasure for the suppressive effect of HH; and (d) other substances that promote macrophage infiltration into injured tissue are effective HH countermeasures.

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APPENDIX
M11 Statistical Analysis of Crush Injury at NB and HB Pressures

Analysis Description

This analysis is based on Affymetrix Mouse 1.0 ST arrays that were processed in the DEOHS microarray core in April 2012. Data from these arrays can be summarized at the transcript or probeset (roughly exon) level. For this analysis, we normalized the data using a quantile normalization and then summarized at the transcript level using a robust multi-array average [A-1].

These data are all paired, meaning that for each mouse the right gastrocnemius muscle was injured whereas the left gastrocnemius was not. Because of this experimental design, we are forced in some situations to make paired comparisons.

Normally, if the injured and uninjured muscles came from different mice, the numerator of a t-statistic would consist of the mean expression of the injured samples minus the mean expression of the uninjured samples. But we can only do this if the injured and uninjured samples are independent. Since each pair of samples came from an individual mouse, they are not independent (e.g., if mouse 5012 has a cold, some genes may be upregulated in both samples from that mouse, but that has nothing to do with the experiment).

To account for this dependence structure, for each mouse we compute the differences between the injured and uninjured gastrocnemius, on a gene-by-gene basis. By subtracting, we remove any mouse-specific variability but retain injury-specific variability. The numerator of our t-statistic will now be the mean of these differences. Any numerator that is significantly larger or smaller than zero indicates that there was a consistent difference due to the injury, after controlling for any intra-mouse variability.

Please note that any paired difference data will be referenced in the output with a “diff” in the name (e.g., for mouse 5012, we label the values from 5012RG – 5012LG as 5012 diff).

We can use these paired differences for all other comparisons as well. There are two reasons to do so. First, as noted above, these data are intrinsically adjusted for intra-mouse variability, which increases our power to detect differences. Second, by making comparisons with the paired data, we can search for genes of interest with one test rather than two.

As an example, consider the HB versus NB comparison for the 32-hour samples. If we use the paired data, algebraically the comparison is (omitting the 32-hour designation for brevity):

(HB injured – HB uninjured) − (NB injured – NB uninjured)

If this equation sums to zero, it indicates that the gene reacts the same to injury regardless of barometric pressure. Conversely, if the equation does not sum to zero, it indicates that the gene in question reacts differently to injury depending on the barometric pressure.

The same result holds for the inter-time comparisons. For example, comparing the 32-hour versus 48-hour samples that were in the HB chamber (omitting the HB designation):

(injured 32 h – uninjured 32 h) − (injured 48 h – uninjured 48 h)

If this equation sums to zero, then we know that the gene in question is equally differentially expressed at 32 and 48 hours. However, if the equation does not sum to zero, we know that the gene changes expression between the time points.

In addition, we can make one more comparison, known as an interaction, which we cannot make with the unpaired data. This tests for genes that react differently to injury at different barometric pressures at different times. There are any number of scenarios that fulfill this criterion, so I will give some examples.

- A gene that is consistently upregulated at 32 and 48 hours under NB conditions but decreases expression between 32 and 48 hours under HB conditions
- A gene that is not differentially expressed in injured tissue except under HB conditions, but only at 32 hours
- A gene that increases expression between 32 and 48 hours under NB conditions but decreases expression between 32 and 48 hours under HB conditions

To make these comparisons, we fit a weighted linear model with four factor levels and then computed empirical Bayes adjusted contrasts. This is very similar to fitting t-statistics, but we gain power in two different ways. First, by using a weighted linear model, we can automatically up- or down-weight samples based on their similarity to other samples of the same type. By doing so, we can reduce any adverse effect from possible outlier samples without having to arbitrarily exclude them from the analysis. This has been shown to increase power to detect true differences in both real and simulated data sets [A-2].

Second, a contrast is more powerful than a t-statistic because the denominator of the statistic is based on the sums of squares of error (SSE) from the model, rather than the standard error of the mean. The SSE estimates intra-group variability over all groups whereas the standard error of the mean only uses the two groups that are being compared. By using more data to estimate variability, we get a more accurate estimate. In addition, we estimate the overall variability for all genes on the chip and use that value to adjust the SSE for each comparison (this is the empirical Bayes adjustment). Again, by using a variability measure that is based on more data, we can improve the accuracy of our estimate. This has also been shown to increase power to detect true differences in both real and simulated data sets [A-3].

Table A-1 lists the comparisons made and the number of probesets selected at an unadjusted p-value of 0.05 and an absolute fold change of 1.5. These data were output in both HTML and text format, with filenames that describe the comparisons. The index.html file found in this directory contains links to all HTML tables generated during this analysis (simply double-click on that file to open in a browser).

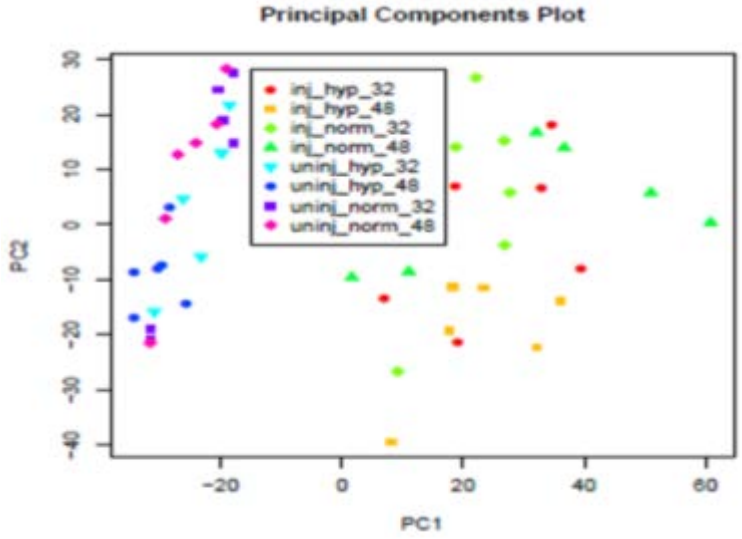
Please note that there is very little evidence for any differential expression in the last five comparisons in Table A-1. If we adjust the *p*-values for multiple comparisons, we do not get any significant genes in any of those comparisons, except for HB 32 hours post vs. HB 48 hours post. This does not mean that there are no differentially expressed genes; we may simply not have enough power to detect differences.

Table A-1. Comparisons and Number of Probesets Selected at an Unadjusted *p*-value < 0.05 and a |fold| >2

Comparisons	Probesets
HB 32 h post injury vs. control	389
HB 48 h post injury vs. control	528
NB 32 h post injury vs. control	359
NB 48 h post injury vs. control	593
HB 32 h post vs. NB 32 h post	46
HB 48 h post vs. NB 48 h post	44
HB 32 h post vs. HB 48 h post	44
HB 32 h post vs. NB 48 h post	73
Interaction	207

QC Plots

Figure A-1 is a principal components analysis (PCA) plot of the unpaired data. PCA is a way to reduce high dimension data to just a few dimensions so we can create plots that show the overall grouping structure. Ideally, samples of the same type will plot together, possibly separated from samples of a different type.



The first principal component (PC1) captures most of the differences between samples. Here we can see that this is due to the injury status. The second principal component (PC2) captures the next largest differences between samples. To a certain extent this appears to be due to the time differences.

Figures A-2 and A-3 show the normalized unscaled standard errors (NUSE) and relative log-scale expression (RLE) plots for these data. For both plots, we want the boxes to line up with each other; in the case of the NUSE plots, we want the data to line up with equal spread across the horizontal line at 1. The same is true for the RLE plot, but across the horizontal line at zero. Samples 4001LG and 4002LG appear to be problematic in Figure A-2. These are the two purple squares on the lower left in Figure A-1. These are well separated from the other uninjured NB 32-hour samples, and one could argue that they should be excluded from the analysis. I re-ran the analysis with these samples excluded, and there was very little difference, so I have retained them for this analysis.

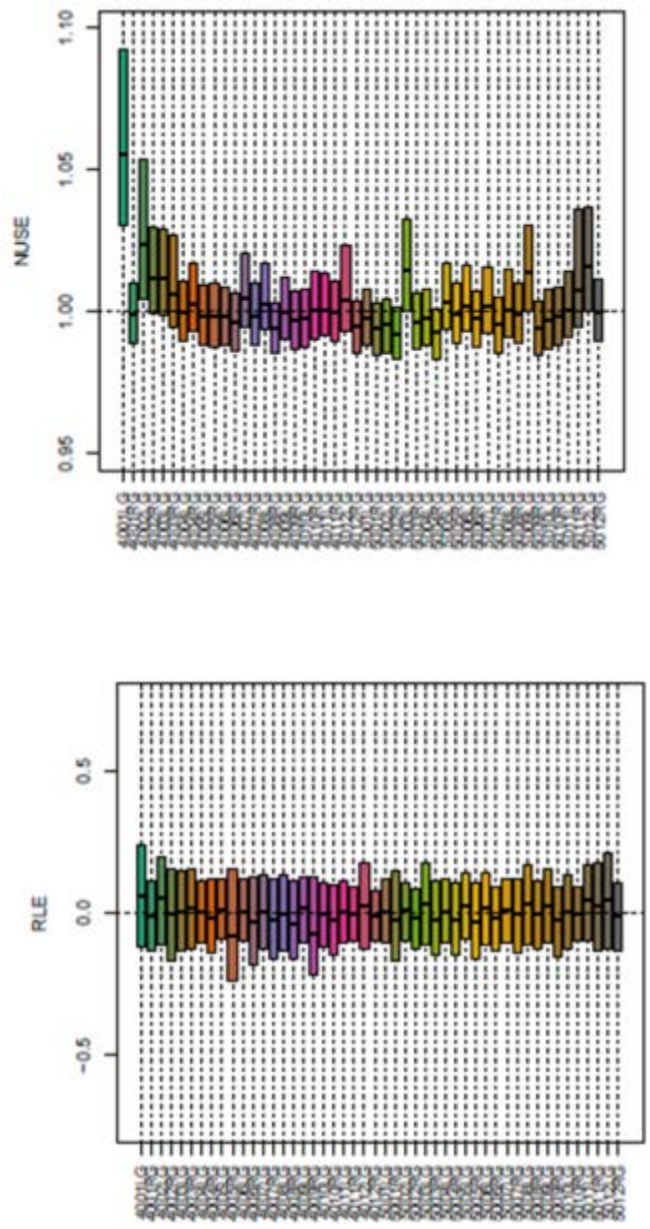


Figure A-3. RLE plot.

Figures A-4 and A-5 are so-called MA plots. On the horizontal axis we plot the log average of the data, and on the vertical axis we plot the log difference between a given sample and the median of all samples. Ideally, the data will cluster in an oval, centered on the horizontal line at zero, which is what we see here.

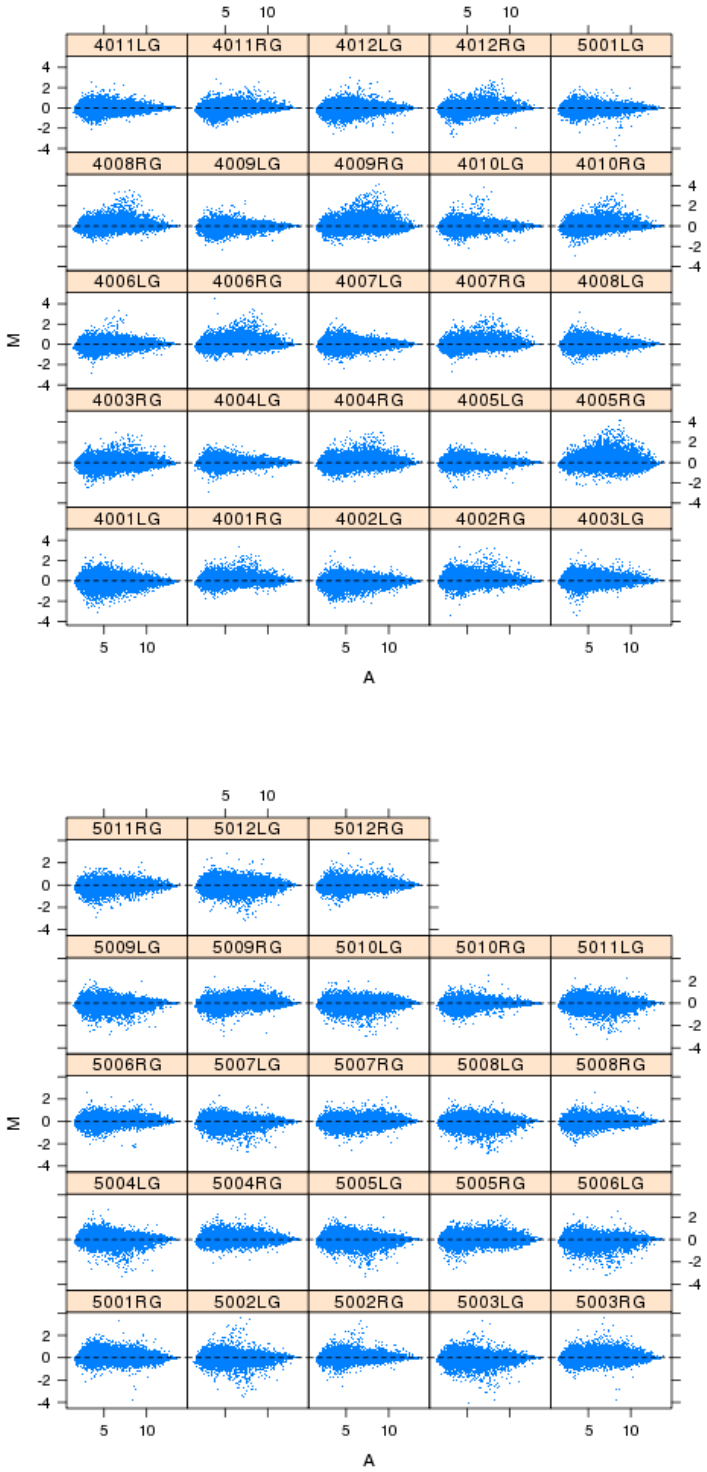


Figure A-5. MA plot, samples 26-48.

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LIST OF ABBREVIATIONS AND ACRONYMS

AOI	area of interest
BW	body weight
CIHB	crush injury + hypobaria
CINB	crush injury + normobaria
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
HB	hypobaric
HH	hypobaric hypoxia
IACUC	Institutional Animal Care and Use Committee
IF	intact female
IGF-1	insulin-like growth factor-1
IL	interleukin
mRNA	messenger ribonucleic acid
NB	normobaric
NUSE	normalized unscaled standard errors
OC	ovariectomized control
OE	ovariectomized estradiol
PBS	phosphate-buffered saline
PCA	principal components analysis
PCR	polymerase chain reaction
RLE	relative log-scale expression
SE	standard error
SHHB	sham + hypobaria
SHNB	sham + normobaria
SSE	sums of squares of error
TNF	tumor necrosis factor
UH	uterine horn
UNLV	University of Nevada, Las Vegas
USAF	United States Air Force